

Phospholipase A₂-driven Phospholipid Metabolism During Phagocytosis

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Phospholipase A₂s generate lipid mediators that constitute an important component of the integrated response of macrophages to stimuli of the innate immune response. Because these cells contain multiple phospholipase A₂ forms, the challenge is to elucidate the roles that each of these forms plays in regulating normal cellular processes and in disease pathogenesis. A major issue is to precisely determine the phospholipid substrates that these enzymes use for generating lipid mediators. There is compelling evidence that group IVA cytosolic phospholipase A₂ (cPLA₂α) targets arachidonic acid-containing phospholipids but the role of the other cytosolic enzyme present in macrophages, the Ca²⁺-independent group VIA phospholipase A₂ (iPLA₂β) has not been clearly defined. We applied mass spectrometry-based lipid profiling to study the substrate specificities of these two enzymes during inflammatory activation of macrophages with yeast-derived zymosan, a phagocytosable particle. Using selective inhibitors, we find that, contrary to cPLA₂α, iPLA₂β spares arachidonate-containing phospholipids and hydrolyzes only those that do not contain arachidonate, in particular choline phospholipids containing palmitic acid at the sn-1 position. This in turn results in the liberation of palmitoleic acid, which is incorporated into inositol lipids. The biological significance of this novel iPLA₂β-driven pathway will be discussed.

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As many of you know, I was a postdoc with Ed in the 90's and spent many years here in la Jolla, so I thought it would be good to start my talk by showing the very first picture I have with Ed. This is the Fall of 93, that is 23 years ago. Obviously, I look much younger, but if you look at Ed, you'll notice that he looks today very much as he looked 23 years ago... ([Slide 2](#)). Well, scientifically-wise, if I had to summarize my years at UCSD with just one image, this would be it ([Slide 3 – Distinct Roles in Signal Transduction](#)). This is the scheme we worked on for all those years to define the roles of multiple phospholipase A₂s in arachidonic acid mobilization in macrophages; cPLA₂ as the key enzyme, sPLA₂ in an amplifying role, and iPLA₂ helping replenish the AA pools by regulating lyso availability. I know Ed does not use it anymore and I don't either, but we published it in a number of papers over the years, and we even got it on the cover of JBC ([Slide 4](#)). Most of these studies with Ed focused to PLA₂-mediated AA mobilization in macrophages, so today I'm going to talk about phospholipids that do not have AA, what happens to them during macrophage activation ([Slide 5 – What About Phospholipids Without Arachidonate?](#)). To study this we used the following protocol ([Slide 6 – Lipidomic Profiling of Phospholipids Without AA](#)). This figure shows a mass spec analysis of major phosphatidylcholine (PC) species after zymosan activation, and the effect of the PLA₂ inhibitors FKGK18 (iPLA₂β) and pyrrophenone (cPLA₂α) ([Slide 7 – Major PC Species Without AA in Zymosan-stimulated Macrophages](#)). We found quite significant decreases in some of these phospholipids, indicating that not only AA is released during activation but other fatty acids follow the same fate. Interestingly, FKGK18 but not pyrrophenone was able to prevent these decreases, suggesting a role for iPLA₂β. The most prominent effects were found in the two major species measured –PC(32:0) and PC(34:1)–, the levels of which were completely restored in the presence of FKGK18. These two species could be identified as PC(16:0/16:0) and PC(16:0/18:1) in fragmentation experiments. Minor species whose hydrolysis was completely prevented by FKGK18 were PC(32:1) and

PC(34:2), and these also could be identified by fragmentation experiments as PC(16:0/16:1) and PC(16:0/18:2). Therefore, it is striking that four phospholipids identified as iPLA₂β substrates during zymosan activation all contain palmitic acid (16:0) esterified at the sn-1 position. Actually when we measured lysolipid production in activated macrophages, palmitoyl-lysoPC was the major species produced and this production was strongly blocked by FKGGK18, again suggesting that iPLA₂β may be a major contributor to this accumulation (Slide 8 – Lysophospholipid Production in Zymosan-stimulated Macrophages). Note in contrast the other major lysolipid produced in the activated macrophages, stearoyl-lysoPC, whose levels are affected little by FKGGK18 but strongly by pyrrophenone, suggesting a link of this species to cPLA₂α. So in the end, as a conclusion of these experiments, in activated cells, iPLA₂β displays some sort of specificity for PC molecules containing palmitic at the sn-1 position (Slide 9 – iPLA₂ May Have Preference for sn-1 palmitoyl PC).

When we examined the inositol lipids we found a few of them that showed an unexpected behavior, as they increased instead of going down. However the increase was partially prevented by FKGGK18 but not by pyrrophenone, suggesting that iPLA₂β may be involved (Slide 10 – Minor PI Species Without AA in Zymosan-stimulated Macrophages). These species have in common that all possess a 16:1 fatty acid residue. And it is interesting to note that two of them, the two that increase the most, were not found in resting cells, suggesting that if the cells make them, they must have some biological function. So the question is, where does 16:1-PI come from? (Slide 11 – Where Does 16:1-PI Come from?). We measured all the phospholipids that have 16:1 and this is what we found (Slide 12 – 16:1-containing Phospholipids in Resting Macrophages). Most of the fatty acid is in PC, especially in one species, PC(16:0/16:1), while all other classes contained lesser amounts. Now we remind you that this PC species is one of those that I shown in the previous slide that was hydrolyzed by iPLA during activation to give rise to palmitoyl-lysoPC. So we measured the levels of the 16:1-containing phospholipids and found that PC(16:0/16:1) was the only that decreased and the ones that increased were the two preexisting PI molecules plus the 2 newly-formed (Slide 13 – 16:1-containing Phospholipids in Stimulated Macrophages). So, it is likely that much of the 16:1 fatty acid accumulating in PI comes from PC in a reaction that is sensitive to FKGGK18 and thus probably mediated by iPLA₂β (Slide 14 – Where Does 16:1-PI Come from? -PC(16:0/16:1)). One problem with this explanation is that the amount lost from PC is generally less than the amount gained by the PIs. Thus there is possibly a second source for 16:1 for PI. We measured the distribution of 16:1 among lipid classes, phospholipids and neutral lipids by GC/MS, and were surprised to find that depending on the lipid class measured, there were two peaks of 16:1 (phospholipids) or only one (neutral lipids) (Slide 15 – Two 16:1 Isomers in Macrophages). Comparison with commercial standards indicated that one was 16:1n-7 or palmitoleic acid proper and the other could either be 16:1n-10 (sapienic acid) or 16:1n-9 (Slide 16 – Two 16:1 Isomers in Macrophages). We made the DMOX derivative and analyzed it by electron impact MS. Confirming it was actually 16:1n-9, a relatively unusual fatty acid (Slide 17 – The Second Isomer is 16:1n-9). As commented before, there is a fundamental difference in the distribution of these two isomers among lipid classes, the n-9 isomer localizes in both phospholipid and neutral lipids, whereas n-7 only appears in phospholipid (Slide 18 – Distribution of 16:1 Isomers Between Lipid Classes). This distribution of n-9 is quite peculiar since no other fatty acid distributes similarly (Slide 19 – Distribution of 16:1 Isomers Between Lipid Classes). When we analyzed the distribution of n-9 in zymosan-treated cells, we found that it increases in all lipid classes but the highest increases are in neutral lipids (Slide 20 – Distribution of 16:1 Isomers Between Lipid Classes).

The unique distribution of 16:1n-9 among cellular lipids and the finding that its levels are increased during cellular activation suggest a specific biological role for this unusual fatty acid. To study this question, we prepared cells enriched in this fatty acid by incubating them with 10 μM 16:1n-9 for 2 h in serum-free medium. This procedure results in the cells taking up the fatty acid and preferentially accumulating it in neutral lipids, in a similar manner as if they had been previously activated with a receptor-directed stimulus. Then, the cells were stimulated with LPS and the effects on the expression of a number of proinflammatory genes was investigated (Slide 21 – 16:1n-9 Possesses Anti-inflammatory Properties *in vitro*). Cells enriched in 16:1n-9 showed

significant decreases in the expression of all genes tested, and such decreases were generally comparable to those found in the 22:6n-3-treated cells. 16:1. 16:1n-9 was significantly more potent than 16:1n-7 for all genes tested; 16:1n-7 had significant effects only in two of them, *Tnf* and *Nos2*. These data show that 16:1n-9 has a spectrum of biological activity that is clearly distinguishable from that of 16:1n-7. We also conducted experiments with mice (Slide 22 – 16:1n-9 Possesses Anti-inflammatory Properties *in vivo*). In these experiments, the fatty acid was administered i.p. to mice 1 h before i.p. injection of LPS for 6 h. Afterward, the animals were sacrificed, peritoneal cells were harvested, cell samples matched by protein content, and the expression levels of *Il6* were studied. Both 16:1n-9 and 22:6n-3 inhibited *Il6* gene expression by the peritoneal cells isolated after the LPS challenge. Analysis of serum IL-6 protein confirmed a strong decrease in the amount of circulating IL-6 protein in the 16:1n-9-treated mice. Unexpectedly, IL-6 protein levels in serum from 22:6n-3 treated cells were no different from those in serum from control untreated animals.

So, in conclusion, these results suggest that the relatively uncommon fatty acid 16:1n-9 may possess anti-inflammatory activity that could be comparable to that of omega-3 fatty acids, and clearly distinguishable from that of palmitoleic acid. From a biochemical point of view this fatty acid shows unique characteristics, such as its accumulation in PI molecules (perhaps via an iPLA₂β-mediated pathway) and also in neutral lipids. Well I started with my very first pic with Ed during my stay at UCSD and would like to end with the last picture I have with him from these wonderful years (Slide 24 – The Dennis Group 2000). This is the summer of 2000 I believe, a few weeks before I left the lab. You can see that Ed, of course, looks the same as he looks today. Just to conclude, the Acknowledgments slide (Slide 25 – Acknowledgments).

REFERENCES

1. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase A₂ enzymes present in P388D₁ macrophages. *J. Biol. Chem.* 271: 6758–6765.
2. Balsinde, J., and E. A. Dennis. 1997. Function and inhibition of intracellular calcium-independent phospholipase A₂. *J. Biol. Chem.* 272: 16069–16072.
3. Balsinde, J., M. V. Winstead, and E. A. Dennis. 2002. Phospholipase A₂ regulation of arachidonic acid mobilization. *FEBS Lett.* 531: 2–6.
4. Gil-de-Gómez, L., A. M. Astudillo, C. Guijas, V. Magrioti, G. Kokotos, M. A. Balboa, and J. Balsinde. 2014. Cytosolic group IVA and calcium-independent group VIA phospholipase A₂s act on distinct phospholipid pools in zymosan-stimulated mouse peritoneal macrophages. *J. Immunol.* 192: 752–762.
5. Astudillo, A. M., D. Balgoma, M. A. Balboa, and J. Balsinde. 2012. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta* 1821: 249–256.
6. Pérez-Chacón, G., A. M. Astudillo, D. Balgoma, M. A. Balboa, and J. Balsinde. 2009. Control of free arachidonic acid levels by phospholipases A₂ and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 1791: 1103–1113.
7. Balsinde, J., M.A. Balboa, and E.A. Dennis. 2000. Identification of a third pathway for arachidonic acid mobilization and prostaglandin production in activated P388D₁ macrophage-like cells. *J. Biol. Chem.* 275: 22544–22549.
8. Balsinde, J., M. A. Balboa, S. Yedgar, and E. A. Dennis. 2000. Group V phospholipase A₂-mediated oleic acid mobilization in lipopolysaccharide-stimulated P388D₁ macrophages. *J. Biol. Chem.* 275: 4783–4786.
9. Pindado, J., J. Balsinde, and M. A. Balboa. 2007. TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase 2/cyclooxygenase-2 pathway. *J. Immunol.* 179: 4821–4828.

10. Ruipérez, V., J. Casas, M. A. Balboa, and J. Balsinde. 2007. Group V phospholipase A₂-derived lysophosphatidylcholine mediates cyclooxygenase-2 induction in lipopolysaccharide-stimulated macrophages. *J. Immunol.* 179: 631–638.
11. Ruipérez, V., A. M. Astudillo, M. A. Balboa, and J. Balsinde. 2009. Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A₂s. *J. Immunol.* 182: 3877–3883.
12. Pérez-Chacón, G., A. M. Astudillo, V. Ruipérez, M. A. Balboa, and J. Balsinde. 2010. Signaling role for lysophosphatidylcholine acyltransferase 3 in receptor-regulated arachidonic acid reacylation reactions in human monocytes. *J. Immunol.* 184: 1071–1078.
13. Balsinde, J. 2002. Roles of various phospholipases A₂ in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. *Biochem. J.* 364: 695–702.
14. Balsinde, J., and M.A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A₂ in activated cells. *Cell. Signal.* 17: 1052–1062.
15. Pérez, R., M. A. Balboa, and J. Balsinde. 2006. Involvement of group VIA calcium-independent phospholipase A₂ in macrophage engulfment of hydrogen peroxide-treated U937 cells. *J. Immunol.* 176: 2555–2561.
16. Balgoma, D., O. Montero, M. A. Balboa, and J. Balsinde. 2008. Calcium-independent phospholipase A₂-mediated formation of 1,2-diarachidonoylglycerophosphoinositol in monocytes. *FEBS J.* 275: 6180–6191.
17. Balgoma, D., A. M. Astudillo, G. Pérez-Chacón, O. Montero, M. A. Balboa, and J. Balsinde. 2010. Markers of monocyte activation revealed by lipidomic profiling of arachidonic acid-containing phospholipids. *J. Immunol.* 184: 3857–3865.
18. Astudillo, A. M., G. Pérez-Chacón, D. Balgoma, L. Gil-de-Gómez, V. Ruipérez, C. Guijas, M. A. Balboa, and J. Balsinde. 2011. Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells. *Biochim. Biophys. Acta* 1811: 97–103.
19. Valdearcos, M., E. Esquinas, C. Meana, L. Gil-de-Gómez, C. Guijas, J. Balsinde, and M. A. Balboa. 2011. Subcellular localization and role of lipin-1 in human macrophages. *J. Immunol.* 186: 6004–6013.
20. Astudillo, A. M., G. Pérez-Chacón, C. Meana, D. Balgoma, A. Pol, M. A. Del Pozo, M. A. Balboa, and J. Balsinde. 2011. Altered arachidonate distribution in macrophages from caveolin-1 null mice leading to reduced eicosanoid synthesis. *J. Biol. Chem.* 286: 35299–35307.
21. Guijas, C., A. M. Astudillo, L. Gil-de-Gómez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2012. Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages: comparison with arachidonic acid. *Biochim. Biophys. Acta* 1821: 1386–1393.
22. Gil-de-Gómez, L., A. M. Astudillo, C. Meana, J. M. Rubio, C. Guijas, M. A. Balboa, and J. Balsinde. 2013. A phosphatidylinositol species acutely generated by activated macrophages regulates innate immune responses. *J. Immunol.* 190: 5169–5177.
23. Pérez, R., X. Matabosch, A. Llebaria, M.A. Balboa, and J. Balsinde. 2006. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J. Lipid Res.* 47: 484–491.
24. Balsinde, J., B. Fernández, and E. Diez. 1990. Regulation of arachidonic acid release in mouse peritoneal macrophages. The role of extracellular calcium and protein kinase C. *J. Immunol.* 144: 4298–4304.
25. Balsinde, J., B. Fernández, J.A. Solís-Herruzo, and E. Diez. 1992. Pathways for arachidonic acid mobilization in zymosan-stimulated mouse peritoneal macrophages. *Biochim. Biophys. Acta* 1136: 75–82.
26. Balboa, M. A., R. Pérez, and J. Balsinde. 2008. Calcium-independent phospholipase A₂ mediates proliferation of human promonocytic U937 cells. *FEBS J.* 275: 1915–1924.
27. Guijas, C., G. Pérez-Chacón, A. M. Astudillo, J. M. Rubio, L. Gil-de-Gómez, M. A. Balboa, and J. Balsinde. 2012. Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic

- phospholipase A₂-dependent synthesis of lipid droplets in human monocytes. *J. Lipid Res.* 53: 2343–2354.
28. Balsinde, J., B. Fernández, and J.A. Solís-Herruzo. 1994. Increased incorporation of arachidonic acid into phospholipids in zymosan-stimulated mouse peritoneal macrophages. *Eur. J. Biochem.* 221: 1013–1018.
29. Balsinde, J., I.D. Bianco, E.J. Ackermann, K. Conde-Frieboes, and E.A. Dennis. 1995. Inhibition of calcium-independent phospholipase A₂ prevents arachidonic acid incorporation and phospholipid remodeling in P388D₁ macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 92: 8527–8531.
30. Balsinde, J., M.A. Balboa, and E.A. Dennis. 1997. Antisense inhibition of group VI Ca₂₊-independent phospholipase A₂ blocks phospholipid fatty acid remodeling in murine P388D₁ macrophages. *J. Biol. Chem.* 272: 29317–29321.
31. Balboa, M. A., Y. Sáez, and J. Balsinde. 2003. Calcium-independent phospholipase A₂ is required for lysozyme secretion in U937 promonocytes. *J. Immunol.* 170: 5276–5280.
32. Pérez, R., R. Melero, M.A. Balboa, and J. Balsinde. 2004. Role of group VIA calcium-independent phospholipase A₂ in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. *J. Biol. Chem.* 279: 40385–40391.
33. Balboa, M. A., and J. Balsinde. 2002. Involvement of calcium-independent phospholipase A₂ in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J. Biol. Chem.* 277: 40384–40389.
34. Balboa, M. A., R. Pérez, and J. Balsinde. 2003. Amplification mechanisms of inflammation: paracrine stimulation of arachidonic acid mobilization by secreted phospholipase A₂ is regulated by cytosolic phospholipase A₂-derived hydroperoxyeicosatetraenoic acid. *J. Immunol.* 171: 989–994.
35. Diez, E., J. Balsinde, M. Aracil, and A. Schüller. 1987. Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim. Biophys. Acta* 921: 82–89.
36. Guijas, C., J. P. Rodríguez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2014. Phospholipase A₂ regulation of lipid droplet formation. *Biochim. Biophys. Acta* 1841: 1661–1671.
37. Meana, C., L. Peña, G. Lordén, E. Esquinas, C. Guijas, M. Valdearcos, J. Balsinde, and M. A. Balboa. 2014. Lipin-1 integrates lipid synthesis with proinflammatory responses during TLR activation in macrophages. *J. Immunol.* 193: 4614–4622.
38. Rubio, J. M., J. P. Rodríguez, L. Gil-de-Gómez, C. Guijas, M. A. Balboa, and J. Balsinde. 2015. Group V secreted phospholipase A₂ is up-regulated by interleukin-4 in human macrophages and mediates phagocytosis via hydrolysis of ethanolamine phospholipids. *J. Immunol.* 194: 3327–3339.
39. Valdearcos, M., E. Esquinas, C. Meana, L. Peña, L. Gil-de-Gómez, J. Balsinde., and M. A. Balboa. 2012. Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *J. Biol. Chem.* 287: 10894–10904.
40. Balsinde, J., and E. A. Dennis. 1996. The incorporation of arachidonic acid into triacylglycerol in P388D₁ macrophage-like cells. *Eur. J. Biochem.* 235: 480–485.